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14. ABSTRACT
A major impediment to effective prostate cancer treatment involves the acquired resistance to cytotoxic therapies. Components of the tissue microenvironment are increasingly recognized to profoundly influence tumor cell phenotypes that include susceptibilities to toxic insults. Using a genome-wide analysis of transcriptional responses to genotoxic stress induced by cancer therapeutics, we have identified a spectrum of secreted proteins derived from the tumor microenvironment (TME) that have the potential to modify tumor growth and enhance resistance to DNA-damaging cancer therapeutics. These results suggest a mechanism by which genotoxic therapies given in a cyclical fashion can enhance subsequent treatment resistance through cell non-autonomous effects contributed by the TME. To date, the contributions of individual members of this DNA Damage-associated Secretory Program (DDSP) have not been defined, nor have the signaling mechanisms responsible for propagating the DNA-damage signal(s) been determined. Our objective during this grant period is to test whether treatment-associated DNA damage responses in cells comprising the prostate TME promote tumor growth and subsequent therapy resistance. During this funding period we have: (1) Generated a prostate fibroblast cell line stably expressing SPINK1; (2) Evaluated the impact which SPINK1 activation has upon the growth characteristics of prostate cancer cells lines; (3) Examined how SPINK1 secretion from the microenvironment modulates the response of prostate cancer cells to chemotherapeutics: (4) Begun the evaluation of SPINK1 regulatory pathway.

15. SUBJECT TERMS
Prostate cancer, Microenvironment, DNA Damage-associated Secretory Program, SPINK1

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Introduction

A major impediment to effective prostate cancer treatment involves the acquired resistance to cytotoxic therapies. Components of the tissue microenvironment are increasingly recognized to profoundly influence tumor cell phenotypes that include susceptibilities to toxic insults. Using a genome-wide analysis of transcriptional responses to genotoxic stress induced by cancer therapeutics, we have identified a spectrum of secreted proteins derived from the tumor microenvironment (TME) that have the *potential* to modify tumor growth and enhance resistance to DNA-damaging cancer therapeutics. These results suggest a mechanism by which genotoxic therapies given in a cyclical fashion can enhance subsequent treatment resistance through cell non-autonomous effects contributed by the TME. To date, the contributions of individual members of this DNA Damage-associated Secretory Program (DDSP) have not been defined, nor have the signaling mechanisms responsible for propagating the DNA-damage signal(s) been determined. The research supported by this award aims to test whether treatment-associated DNA damage responses in cells comprising the prostate TME promote tumor growth and subsequent therapy resistance. Our specific aims are as follows: (1) Determine the contribution of specific effectors of the tumor microenvironment-derived DDSP in modulating resistance to cytotoxic chemotherapy and ionizing radiation; (2) Determine the mechanism(s) by which the DDSP is activated; (3) Determine if therapeutic targeting of SPINK1 in the TME can attenuate therapy resistance.

Body

The following summarizes the research accomplishments in the first year of this proposal, as associated with each task in the Statement of Work.

Task 1: Determine the contribution of specific effectors of the tumor microenvironment-derived DDSP (e.g. SPINK1) in modulating resistance to cytotoxic chemotherapy and ionizing radiation. (Months 1-10).

We choose to focus on SPINK1 for this task based on the following reasons: First, we found SPINK1 to be highly induced (~20-fold) in the TME following chemotherapy (1); Second, SPINK1 is secreted and was recently shown to engage the EGFR pathway and promote cell proliferation (2); Third, antibodies to SPINK1 have been shown to be effective in blocking SPINK1 activity, thus providing a clear translational pathway (2); Fourth, while SPINK1 is overexpressed by a minority (about 10% of prostate cancers) (3), our data indicate that SPINK1 may be a highly relevant target in most prostate cancer patients that undergo treatment with genotoxic agents such as radiation or chemotherapy.

Task 1a: Generation of stable PSC27 cell lines both overexpressing SPINK1 and with SPINK1 silenced. Complete confirmation experiments of altered SPINK1 expression using western blot, quantitative PCR and immunofluorescence. (Months 1-3).

Lenti-viral particles containing ORF constructs coding for constitutively active SPINK1 and a GFP-marker were generated and used to transduce PSC27 immortalized prostate fibroblasts cells. Cells were then maintained under the selective pressure of Blasticidin S for several weeks in order to eliminate non-transduced cells resulting in a population stably overexpressing SPINK1 transcript. In parallel PSC27 cells were similarly targeted with lenti-viral particles containing shRNA's targeting SPINK1 transcriptional activity. shRNA targeted

cells were then maintained under Puromycin selective pressure until the population uniformly expressed the GFP-tag. In addition empty-vector PSC27 cell lines for both the overexpression and silenced constructs were generated for use as controls in future experiments. Transcript abundance for all lines was verified using quantitative PCR and protein abundance via western blot analysis (Figure 1). Our results revealed that we were able to stably induce SPINK1 transcript production to levels consistent with those found in prostate fibroblasts following γ-irradiation. Further, we were able to verify that protein was secreted into the conditioned medium. This stable overexpression and secretion was critical for our downstream experiments.

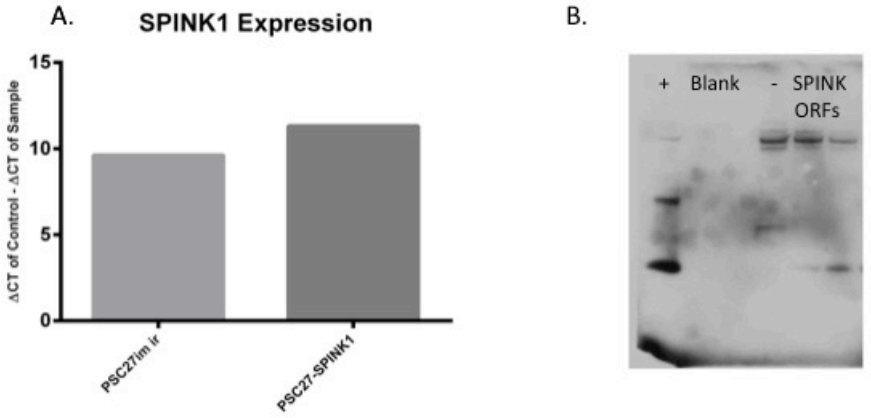

Figure 1. Verification of induced SPINK1 transcript and protein. A: The change in transcript of SPINK1 compared to untreated control cells for both irradiated PSC27im and PSC27im cells transduced with the ORF construct. B: The lower bands on the western blot represent SPINK1 protein detected in the conditioned medium collected from the SPINK1 ORF cell lines along with protein detected in our positive control of pancreatic lysate.

Task 1b: Obtain institutional approval for all proposed animal experiments. (Month 1-4).

All approvals are now in place.

Task 1c: Complete co-culture experiments with prostate cancer cell lines (PC3, DU145, VCaP, 22rv1) and the PSC27^{SPINK1} fibroblasts. Evaluate epithelial cell growth compared to control PSC27 fibroblasts. (Months 3-5).

This task was designed to mimic the growth response of prostate cancer cells when exposed to a SPINK1 activated environment. It further allows us to evaluate how microenvironment signaling can contribute to the growth rates of a primary tumor. To evaluate this response several prostate cancer cell lines were grown with or without presence of overexpressed SPINK1 derived from prostate fibroblast cells. Following 48 hours of treatment total cell numbers were quantitated and compared against controls. These results revealed that SPINK1 overexpression enhanced cell growth in all of the prostate cancer lines evaluated (Figure 2).

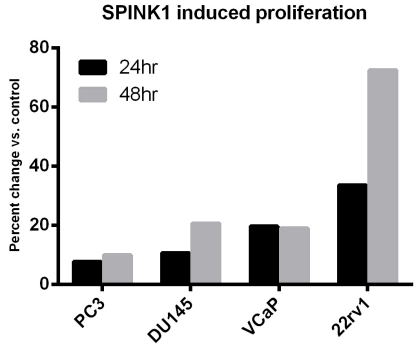

Figure 2. A SPINK1 enriched environment induced increased proliferative activity amongst prostate cancer cell lines. Each cell line was grown in a SPINK1 enriched environment for either 24hrs or 48hrs alongside a matching control. After the prescribed time-period the percent change vs. the control was measured via a MTS assay.

Task 1d: Generation of conditioned media from stable PSC27 cell lines overexpressing SPINK1 and with SPINK1 silenced. (Months 3-5)

We are continually generating fresh conditioned media for each experiment. The media is prepared by adding serum free DMEM to a flask of cells which is ~70% confluent and allowing them to grow for three days. After the prescribe time has elapsed the media is collected and sterile filtered prior to use. The presence of SPINK1 in the conditioned media was verified using western blot analysis and activity confirmed by measuring the phosphorylation of EGFR in treated cancer cell lines.

Task 1e: Complete treatments with conditioned media with on prostate cancer cell lines (PC3, Du145, 22rv1, LNCaP, VCaP). Expose cells to IC50 concentrations of chemotherapeutics and/or ionizing radiation. Following exposure to the cytotoxic therapies quantitate growth characteristics and apoptosis. (Months 5-7).

Prostate cancer cells were exposed to IC50 concentrations of docetaxel and supplemented with conditioned media generated from the SPINK1 overexpressing cell line or the matched control. Cells were allowed to grow for 48hrs in these conditions prior to evaluating growth characteristics and apoptosis. Our results indicate that there were varying effects of SPINK1 on the growth of prostate cells when exposed to docetaxel (Figure 3). Specifically, both PC3 and Du145 cells had a slight increase in cell growth compared to the matched control while Vcap had a modest reduction in cell growth when exposed to CM and docetaxel. No change in cell growth was observed for either 22rv1 or LNCaP cells when compared to controls of the same conditions. When apoptosis was evaluated we found that conditioned media supplemented Du145 and Vcap cells had similar level of apoptosis compared to controls when exposed to docetaxel (Figure 4). In the remaining cell lines, we observed a reduction in apoptosis when supplemented with conditioned medium suggesting that SPINK1 may provide some level of protection to cytotoxic therapies (Figure 4).

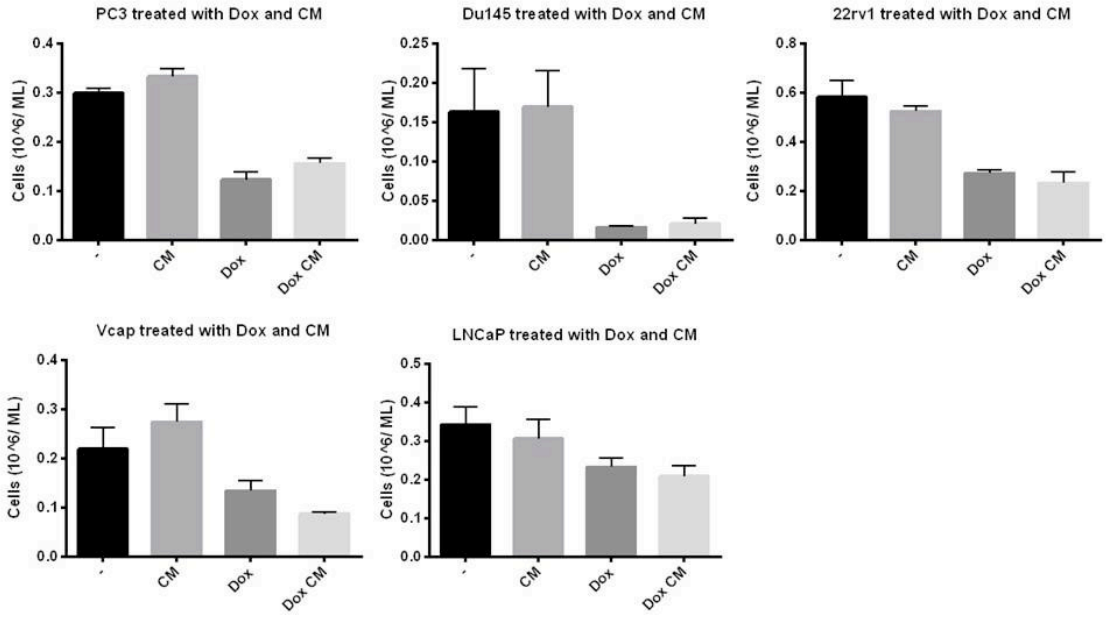

Figure 3. Growth response of prostate cancer cell lines when exposed to docetaxel (Dox) and SPINK1 conditioned medium (CM) for 48 hours. Enhanced cellular growth was observed for PC3 and Du145 cells after Dox treatment when supplement with CM. Surprisingly a reduction in cell growth was detected after Dox treatment with CM supplementation for Vcap cells. While no change in growth rates were observed after treatment for 22rv1 or LNCaP cells.

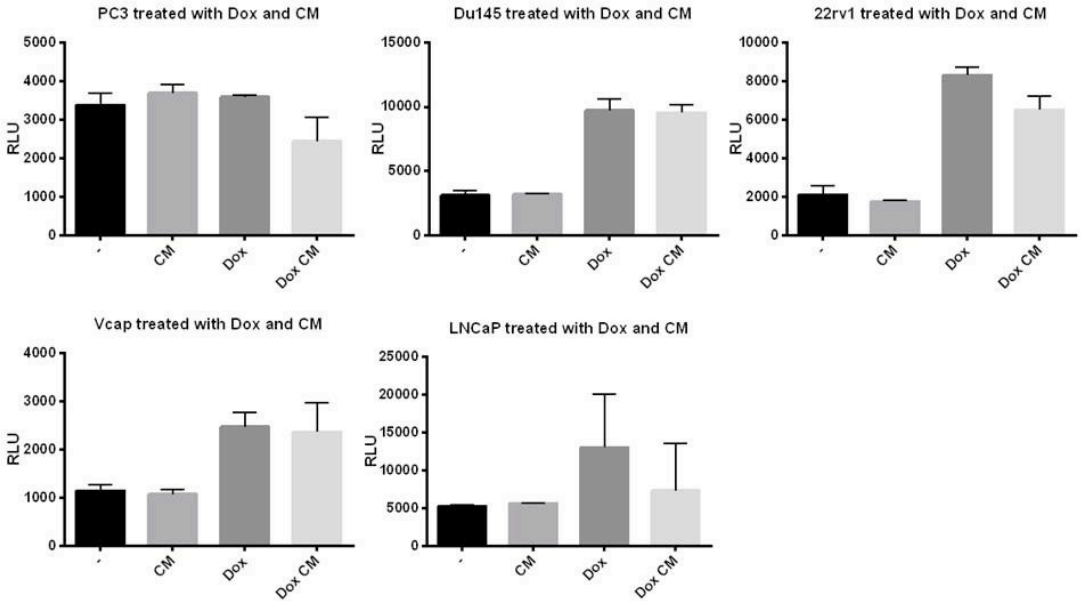

Figure 4. Apoptosis response of prostate cancer cell lines when exposed to docetaxel (Dox) and SPINK1 conditioned medium (CM) for 48 hours. No change in apoptosis was observed for Du145 and Vcap cells after Dox treatment when supplement with CM. In the remaining cell lines a reduction in apoptosis was detected after Dox treatment with CM supplementation compared to the matched controls.

***Task 1f*:** Xenograft implantation of 3 study arms using 10 SCID mice per arm. Arm 1 will combine prostate cancer cells with the $PSC27^{SPINK1}$ fibroblasts and implant the recombinants under the renal capsule of immune-deficient recipient mice. Arm 2 will combine prostate cancer cells with the silenced $PSC27^{SPINK1}$ fibroblasts. Arm 3 will use only prostate cancer cells. (Months 4-10). **Postponed**

> ***Subtask 1:*** Harvest grafts after 8 weeks and compare growth against controls. Perform statistical analysis of outcomes.
>
> ***Subtask 2:*** Assess the histologic appearance of the grafts by H&E staining and evaluate the tissue architecture.

<u>Task 2</u>: Determine the mechanism(s) by which the DDSP is activated. (Months 8-18).

Task 2a: Generate PSC27 prostate fibroblasts cell line with stable expression of a dominant negative NFkB inhibitor (mutant IκBα). (Months 8-10).

The purpose of this task was to investigate the contribution that NFκB signaling has in the activation of SPINK1 signaling. NFkB was targeted given the presence of NFkB binding sites in the promoter region SPINK1. To accomplish this task we generated a PSC27 fibroblast cell line that stably expressed a dominant negative NFkB inhibitor ($PSC27^{I\kappa B\alpha}$). These cells could then be exposed to cytotoxic therapies and compared against control cell lines to establish the importance of NFkB in this signaling pathway.

***Task 2b*:** Expose the PSC27$^{I\kappa B\alpha}$ cells to DNA damage, through ionizing radiation, and quantitate SPINK1 expression. Perform statistical analysis of outcomes. (Months 10-12).

The PSC27$^{I\kappa B\alpha}$ and controls cell lines were exposed to 10 gray of ionizing radiation and then allowed to incubate for 12 days at 37°C. This time-point was chosen after previous experiments indicated that 12 days post irradiation corresponded to peak SPINK1 transcript levels. After the prescribed period of time cells were collected and SPINK1 transcript abundance was measured. Our results revealed that SPINK1 transcript was upregulated following ionizing radiation in both the control and PSC27$^{I\kappa B\alpha}$ cell lines (Figure 5). If NFkB signaling were solely responsible for SPINK1 activation we would expect transcript levels to remain unchanged following the treatment. However, we did detect a slight increase in transcript levels for the PSC27$^{I\kappa B\alpha}$ cells after radiation therapy suggesting that NFkB, while involved, is only one component of the SPINK1 regulatory pathway.

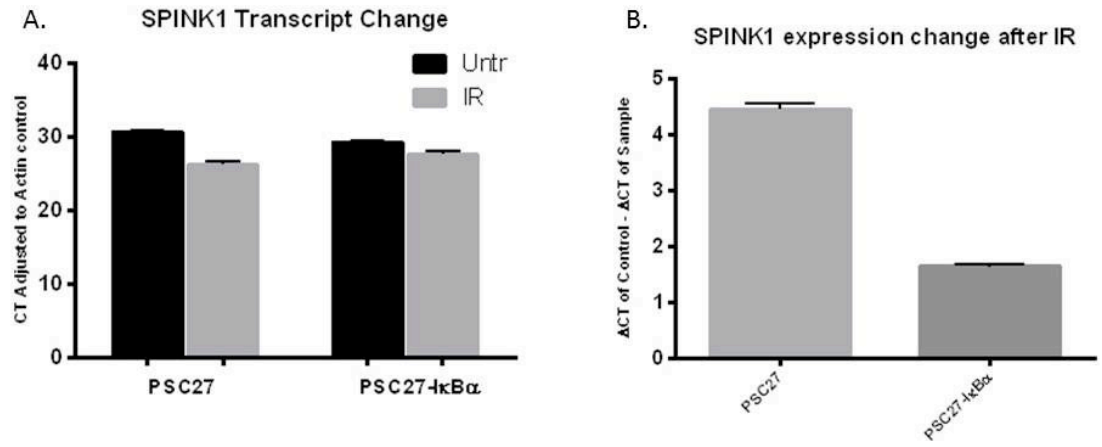

Figure 5. The Quantitative PCR measurement of SPINK1 transcript change in the PSC27$^{I\kappa B\alpha}$ cell line following exposure to 10 gray of ionizing radiation (IR). A: SPINK1 transcript abundance was measured 12 days post IR for both controls and PSC27$^{I\kappa B\alpha}$ cell lines. A smaller CT count indicates increased transcript abundance. B: The difference in CT cycles between the control and PSC27$^{I\kappa B\alpha}$ cell lines pre and post IR. The positive changes indicate an increased abundance of transcript following IR for both cell lines.

Task 2c: Complete the evaluation of NFkB binding to the SPINK1 promoter using a ChIP assay detecting chromatin captured by NFkB antibodies and PCR primers specific to sequences flanking SPINK1 promoter regions before and after ionizing radiation. Perform statistical analysis of outcomes. (Months 12-14). **Pending**

Task 2d: Complete the functional assessment of the NFkB binding sites in the promoter region of SPINK1. (Months 13-18). **In progress**

In order to further evaluate the regulatory pathway of SPINK1 signaling we generated full length and partial sequences of the promoter region. These sequences we designed to encompass different binding site regions of the SPINK1 promoter and therefore be used for our downstream analyses.

Subtask 1: Generate SPINK1 luciferase reporter vectors comprising different regions of the SPINK1 promoter and transfect them into PSC27 cells. (Months 13-16).

To evaluate transcription factor binding that results in the activation of SPINK1, the above-mentioned promoter constructs were cloned into the PGL3 dual-luciferase system. This system includes the PGL3 basic vector encoded with a luciferase reporter and the PGL3 control vector encoded with the rinella reporter. The reporter constructs were simultaneously transiently transfected into PSC27im cells, which were then exposed to various SPINK1 activating treatments. The samples were then processed according the manufactures' protocol and luciferase and rinella activity was evaluated. Rinella represented transfection efficiency and luciferase was a measure of SPINK1 activation. While in most situations we were able to achieve a high efficiency for the transfection, SPINK1 activation was difficult to achieve. We are currently in the process of redesigning the SPINK1 promoter constructs and looking for more consistent activators of SPINK1.

Subtask 2: Complete treatment on luciferase reporter cell lines with TNFα, a known activator of NFkB signaling, and evaluate response. Perform statistical analysis of response. (Months 15-18). **Pending**

<u>Task 3</u>: Determine if therapeutic targeting of SPINK1 in the TME can attenuate therapy resistance. (Months 15-23). Pending

Task 3a: Xenograft implantation study using 11 SCID mice per arm. The first set of arms will combine prostate epithelial tumor cells (PC3; 22rV1; VCaP) with the PSC27 fibroblasts and implant the recombinants into the left flanks of immune-deficient recipient mice. The remaining arms will combine prostate epithelial tumor cells with the silenced $PSC27^{SPINK1}$ fibroblasts and implant the recombinants into the same site as the previous arms. (Months 15-17). **Pending**

Subtask 1: Monitor xenograft tumors until enrollment volume of 400 mm^3 is reached. (Months 16-17).

***Task 3b*:** Complete treatment studies in xenograft mice. Mice will be exposed to systemic DNA damage and given twice-weekly doses of a mAB to SPINK1 (10 mg/kg) or a placebo and treatment effects on tumor volume will be monitored. Perform statistical analysis of outcomes. (Months 17-19). **Pending**

***Task 3c*:** Complete the cell specific evaluation of tumors collected at the end of task 12. (Months 19-23). **Pending**

Subtask 1: Complete IHC and Western analyses for SPINK1 (fibroblasts), Ki67 (epithelium), EGFR (epithelium) and EGFR pathway activation (epithelium) (Months 19-20)

Subtask 2: Complete the isolation of major cell types (epithelium, fibroblasts) from tumors using flow cytometry. (Months 20-21)

Subtask 3: Perform RNA extraction and amplification. Quantitate components of the DDSP and perform data analysis. (Months 21-23)

<u>Task 4</u>: Compile data and complete drafting of manuscript for publication. (Months 20-24). Pending

Key Research Accomplishments

- We have successfully generated prostate fibroblasts that contain SPINK1 overexpression and control constructs. Additionally the upregulation of SPINK1 transcript and protein has been verified for these cell lines.
- Conditioned medium has been generated and secreted functionally active SPINK1 protein has been verified.
- Dual treatment of prostate cancer cell lines with conditioned medium and chemotherapeutics has been completed.
- We have evaluated the SPINK1 promoter region to determine which transcription factor binding sites are present.
- We were able to confirm that NFκB is at least partially involved in the signaling cascade regulating SPINK1 activation via use of the PSC27$^{I\kappa B\alpha}$ cells.
- The full length and partial SPINK1 promoter region has been cloned into the PGL3 dual luciferase system.

Reportable Outcomes

Not applicable at the present time.

Conclusions

In summary, over the past year we have made significant progress toward our goal of understanding the role that SPINK1 plays in the DNA Damage-associated Secretory Program. We have completed the critical step of creating a functionally active stably overexpressed SPINK1 prostate fibroblast line, which serves as the foundation for many of our downstream experiments. We have also established that increased SPINK1 originating from the microenvironment can enhance the proliferative capacity of prostate cancer cells. In addition this SPINK1 rich environment can alter the response of prostate cancer cell lines to the standard of care chemotherapy, docetaxel. This may be particularly relevant finding for patients undergoing treatment for advanced prostate cancer. We have also made headway towards understanding the regulatory pathway of SPINK1 activation. The SPINK1 promoter region has been evaluated and the transcription factor binding sites contained within have been identified. One particular transcription factor of interest (NFκB) with multiple binding sites has been further investigated via the use of a dominant negative NFkB inhibitor cell line. Irradiation of this overexpression line confirmed that NFκB is at least partially involved in the signaling cascade regulating SPINK1 activation.

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